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Appl. No. : **09/700,130**
Filed : **December 18, 2001**

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application.

LISTING OF CLAIMS

Claims 1-99 (Cancelled)

Claim 100 (New) A method of conditionally controlling the survivability of a recombinant microbial cell population, said method comprising:

providing cells of a recombinant microbial cell population, which comprise a gene coding for a gene product of interest, with a gene coding for a cytotoxin polypeptide of a proteic killer gene system, wherein said gene coding for said cytotoxin polypeptide is operably linked to a regulatable, regulatory DNA sequence such that said cytotoxin polypeptide is expressed when cells of said cell population are present in an undesired environment.

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Claim 101 (New) The method according to Claim 100, wherein the cells of said cell population are further provided with a gene coding for an antidote polypeptide of said proteic killer gene system, wherein said antidote polypeptide binds to said cytotoxin polypeptide, thereby resulting in at least a partial counteraction of the toxic effect of said cytotoxin polypeptide.

Claim 102 (New) The method according to Claim 101, wherein the gene coding for said antidote polypeptide that binds to said cytotoxin polypeptide of said proteic killer gene system is operably linked to a regulatable, regulatory DNA sequence such that said gene coding for said antidote polypeptide is suppressed under conditions whereby said gene coding for said cytotoxin polypeptide is expressed.

Claim 103 (New) The method according to Claim 101, wherein the expression of said genes coding for said cytotoxin and/or antidote polypeptide of said proteic killer gene system is stochastically regulated.

Claim 104 (New) The method according to Claim 103, wherein said stochastical regulation is effected by operably linking said genes coding for said cytotoxin and/or

antidote polypeptide of said proteic killer gene system to a regulatory sequence that comprises an invertible promoter.

Claim 105 (New) The method according to Claim 103, wherein said stochastical regulation is effected by flanking at least part of said regulatory sequence by repeat sequences such that at least part of said regulatory sequence is then recombinationally excised.

Claim 106 (New) The method according to Claim 100, wherein at least one of said genes of said proteic killer gene system is present on the chromosome of said cell.

Claim 107 (New) The method according to Claim 100, wherein at least one of said genes of said proteic killer gene system is present on an extrachromosomal replicon.

Claim 108 (New) The method according to Claim 100, wherein said cytotoxin polypeptide inhibits translation.

Claim 109 (New) The method according to Claim 100, wherein said genes of said proteic killer gene system are derived from Gram-negative bacteria, Gram-positive bacteria or from a species belonging to the *Archae*.

Claim 110 (New) The method according to Claim 100, wherein said Gram-negative bacteria are selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

Claim 111 (New) The method according to Claim 100, wherein said Gram-positive bacteria are selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

Claim 112 (New) The method according to Claim 111, wherein said Gram-positive bacterium is *Bacillus thuringiensis*.

Claim 113 (New) The method according to Claim 100, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution,

deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 114 (New) The method according to Claim 100, wherein said regulatory DNA sequence regulates the expression of said gene coding for a cytotoxin polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultivation medium.

Claim 115 (New) The method according to Claim 114, wherein said promoter is inducible by a chemical compound.

Claim 116 (New) The method according to Claim 114, wherein said promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound such that when said first kind of compound is depleted from the medium, the promoter is induced by said second kind of compound.

Claim 117 (New) The method according to Claim 100, wherein said gene product of interest is an immunologically active gene product.

Claim 118 (New) The method according to Claim 100, wherein said gene product of interest is one that is effective in degradation of an environmental pollutant.

Claim 119 (New) The method according to Claim 100, wherein said gene product of interest is a pesticidally active product.

Claim 120 (New) The method according to Claim 119, wherein said gene coding for said pesticidally active gene product is derived from *Bacillus thuringiensis*.

Claim 121 (New) A method of limiting the survival of a cell population in a first or a second environment, said method comprising:

(i) providing cells of a cell population, which comprise a gene coding for a gene product of interest, with a gene coding for a cytotoxin polypeptide of a proteic killer gene system, wherein said gene coding for said cytotoxin polypeptide is operably linked to a regulatory DNA sequence which is regulatable by an environmental factor that regulates the expression of said gene coding for said cytotoxin polypeptide, and wherein said cell population is present under environmental conditions whereby said gene coding for said cytotoxin polypeptide is expressed such that said expression results in at least partial killing of said cell population.

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Claim 122 (New) The method according to Claim 121, wherein the survival of said cell population is limited in a first environment in which said gene coding for said cytotoxin polypeptide is expressed, said cell population thereby being contained in said first environment.

Claim 123 (New) The method according to Claim 121, wherein the survival of said cell population is not limited when present in a first environment wherein said gene coding for said cytotoxin polypeptide is not expressed, the survival of said cell population being limited when present in a second environment or when said first environment is physically or chemically changed, such that said gene coding for said cytotoxin polypeptide is expressed.

Claim 124 (New) The method according to Claim 121, wherein said gene coding for said cytotoxin polypeptide is operably linked to a nucleotide sequence coding for an antitoxin repressor substance that is capable of undergoing a decay when said cells are present a second environment to an extent that said repressor substance is converted to a non-functional form, thereby resulting in the gradual limitation of the function of the cells of said cell population.

Claim 125 (New) The method according to Claim 121, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 126 (New) A method of stochastically limiting the survival of a cell population in an environment, said method comprising:

providing cells of said cell population with a recombinant replicon comprising a regulatably expressible gene that codes for a cytotoxin polypeptide of a proteic killer gene system, wherein expression of said gene coding for said cytotoxin polypeptide in said

cells leads to formation of said cytotoxin polypeptide to an extent that the function of said cells is limited, and wherein the expression of said gene coding for said cytotoxin polypeptide is stochastically induced by recombinational excision of an excisable negatively functioning regulatory nucleotide sequence that is present in said recombinant replicon which comprises said gene coding for said cytotoxin polypeptide or in an other recombinant replicon which is present in the cells of said cell population, thereby inhibiting expression of said gene coding for said cytotoxin polypeptide.

Claim 127 (New) The method according to Claim 126, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid RI PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 128 (New) A method of post-segregationally stabilizing a plasmid in a recombinant microbial host cell population, said method comprising:

(i) providing cells of a recombinant microbial cell population, which comprise a gene coding for a gene product of interest, with a plasmid which comprises a gene coding for a cytotoxin polypeptide of a proteic killer gene system and a gene coding for the corresponding antidote polypeptide of said proteic killer gene system, wherein said antidote polypeptide is capable of being degraded in cells of said cell population at a higher rate than said corresponding cytotoxin polypeptide; and

(ii) cultivating said cell population under conditions such that said genes of said proteic killer gene system are expressed, wherein a daughter cell that does not receive at least one copy of said plasmid is killed as a result of said faster degradation of said antidote polypeptide.

Claim 129 (New) The method according to Claim 128, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 130 (New) A method of confining an extrachromosomal replicon to a recombinant microbial cell population, said method comprising:

(i) isolating a microbial cell which naturally comprises a gene coding for a cytotoxin polypeptide of a proteic killer gene system or introducing said gene coding for a cytotoxin polypeptide into a cell which does not naturally comprise said gene;

(ii) introducing into said cell an extrachromosomal replicon to be confined, wherein said replicon comprises a gene coding for a gene product of interest and a gene coding for an antidote polypeptide of said proteic killer gene system that binds to said cytotoxin polypeptide, thereby acting as an antitoxin for said cytotoxin polypeptide; and

(iii) cultivating said cell under conditions whereby the gene of said proteic killer gene system is expressed, wherein a daughter cell that does not receive a copy of said extrachromosomal replicon is killed by said cytotoxin polypeptide in the absence of expression of said antidote polypeptide.

Claim 131 (New) The method according to Claim 130, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of

the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 132 (New) The method according to Claim 130, wherein said replicon is a plasmid having a copy number which is in the range of 1-30.

Claim 133 (New) The method according to Claim 130, wherein said replicon is a plasmid having a copy number which is in the range of 1-10.

Claim 134 (New) The method according to Claim 130, wherein said replicon is a plasmid having a copy number which is in the range of 1-5.

Claim 135 (New) A method of containing an extrachromosomal recombinant replicon, said method comprising:

providing a cell with a recombinant extrachromosomal replicon comprising a gene whose expression results in the formation of a cytotoxin polypeptide of a proteic killer gene system to an extent that cell function is limited, wherein said cell is modified to have a chromosomal replicon comprising a regulatory nucleotide sequence the gene product of which inhibits (a) the expression of said gene coding for a cytotoxin polypeptide or (b) the cell function-limiting effect of said cytotoxin polypeptide, thereby protecting said cell, and wherein an unmodified cell which comprises said extrachromosomal recombinant replicon is limited in cell function.

Claim 136 (New) The method according to Claim 135, wherein said gene coding for said cytotoxin polypeptide of said proteic killer gene system , when expressed, results in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage P1 Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 137 (New) The method according to Claim 135, wherein said replicon is a plasmid having a copy number which is in the range of 1-30.

Claim 138 (New) The method according to Claim 135, wherein said replicon is a plasmid having a copy number which is in the range of 1-10.

Claim 139 (New) The method according to Claim 135, wherein said replicon is a plasmid having a copy number which is in the range of 1-5.

Claim 140 (New) A recombinant cell comprising a gene coding for a cytotoxin polypeptide of a proteic killer gene system, provided that when said cell is an *E. coli* cell, said gene coding for said cytotoxin polypeptide is not derived from *E. coli* or a prophage thereof.

Claim 141 (New) The cell according to Claim 140, further comprising a gene coding for an antidote polypeptide of said proteic killer gene system, wherein said antidote polypeptide is capable of binding said cytotoxin polypeptide thereby at least partially counteracting the toxic effect of said cytotoxin polypeptide.

Claim 142 (New) The cell according to Claim 141, wherein said genes coding for said cytotoxin polypeptide and/or antidote polypeptide are present on the chromosomes of said cell.

Claim 143 (New) The cell according to Claim 141, wherein at least one of said genes of said proteic killer gene system is present on an extrachromosomal replicon.

Claim 144 (New) The cell according to Claim 141, wherein a gene coding for said antidote polypeptide, which binds to said cytotoxin polypeptide of a proteic killer gene system, is operably linked to a regulatable regulatory DNA sequence such that said gene coding for said antidote polypeptide is suppressed under conditions whereby said gene coding for said cytotoxin polypeptide is expressed.

Claim 145 (New) The cell according to Claim 141, wherein the expression of said genes coding for said cytotoxin and/or antidote polypeptide of said proteic killer gene system is stochastically regulated.

Claim 146 (New) The cell according to Claim 145, wherein said stochastical regulation is effected by operably linking said genes coding for said cytotoxin and/or antidote

polypeptide of said proteic killer gene system to a regulatory sequence that comprises an invertible promoter.

Claim 147 (New) The cell according to Claim 145, wherein said stochastical regulation is effected by flanking at least part of said regulatory sequence by repeat sequences such that at least part of said regulatory sequence is then recombinationally excised.

Claim 148 (New) The cell according to Claim 140, wherein said cytotoxin polypeptide inhibits gene translation.

Claim 149 (New) The cell according to Claim 140, wherein said genes of said proteic killer gene system are derived from Gram-negative bacteria, Gram-positive bacteria or from a species belonging to the *Archae*.

Claim 150 (New) The cell according to Claim 149, wherein said Gram-negative bacteria are selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

Claim 151 (New) The cell according to Claim 149, wherein said Gram-positive bacteria are selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

Claim 152 (New) The cell according to Claim 151, wherein said Gram-positive bacterium is *Bacillus thuringiensis*.

Claim 153 (New) The cell according to Claim 140, wherein said genes coding for said cytotoxin polypeptide of said proteic killer gene system, when expressed, result in the formation of a cytotoxin polypeptide selected from the group consisting of an *E. coli* K-12 relE polypeptide, an *E. coli* plasmid P307 relE polypeptide, a plasmid F CcdB polypeptide, a plasmid Rl PemK polypeptide, a plasmid RP4 ParE polypeptide, a prophage Pl Doc polypeptide, a *Streptococcus pneumoniae* cytotoxin polypeptide, an Archeon *Methanococcus janashii* cytotoxin polypeptide and a functionally equivalent polypeptide which is a derivative of any of the aforementioned polypeptides, the sequence of which has been modified by substitution, deletion or addition of one or more amino acids while the gene product of which has retained at least part of the function of the gene product of the non modified sequence.

Claim 154 (New) The cell according to Claim 140, wherein said gene coding for said cytotoxin polypeptide is operably linked to a regulatable, regulatory DNA sequence.

Claim 155 (New) The cell according to Claim 154, wherein said regulatory DNA sequence regulates the expression of said gene coding for said cytotoxin polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultivation medium.

Claim 156 (New) The cell according to Claim 155, wherein said promoter is inducible by a chemical compound.

Claim 157 (New) The cell according to Claim 155, wherein said promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound such that when said first kind of compound is depleted from the medium, the promoter is induced by said second kind of compound.

Claim 158 (New) The cell according to Claim 140, wherein said cell further comprises a gene coding for a gene product of interest.

Claim 159 (New) The cell according to Claim 158, wherein said gene product of interest is an immunologically active gene product.

Claim 160 (New) The cell according to Claim 158, wherein said gene product of interest is one that is effective in degradation of an environmental pollutant.

Claim 161 (New) The cell according to Claim 158, wherein said gene product of interest is a pesticidally active product.

Claim 162 (New) The cell according to Claim 161, wherein said gene coding for said pesticidally active gene product is derived from *Bacillus thuringiensis*.

Claim 163 (New) The cell according to Claims 140, where said cell is selected from the group consisting of Archae, yeast cells, fungal cells, animal cells and plant cells.

Claim 164 (New) The cell according to Claim 163, wherein said animal cell is selected from the group consisting of a mammal cell, a human cell and an insect cell.

Claim 165 (New) A composition comprising a cell according to Claim 140.